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Release of ferulic acid from plant polysaccharides by ferulic acid esterase from *Streptomyces olivochromogenes*

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Streptomyces olivochromogenes ferulic acid esterase released free ferulic acid from 0-[5-0-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-0-β-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) prepared from wheat bran. The K_m and V_{max} of the reaction were 0·24 mM and 0·134 U mg^{-1}, respectively. The enzyme released 0·25% of the covalently bound ferulic acid from sugar beet pectin both in the presence and in the absence of pectinase.

INTRODUCTION

Ferulic acid and *p*-coumaric acid are common constituents of forage fed to animals, and may represent up to 2·5% (w/w) of cell walls in grasses (Hartley & Jones, 1977). Ferulic acid is ester-linked to arabinose in various plant polysaccharides, such as arabinoxylans and pectins (Mueller-Harvey *et al.*, 1986; Rombouts & Thibault, 1986), and may play a role in cell wall growth and stabilisation (Ishii & Hiroi, 1990).

Wheat bran contains 0·66% (w/w) alkali extractable ferulic acid (Smith & Hartley, 1983) and sugar beet pectin contains up to 0·6% feruloylated groups (Rombouts & Thibault, 1986).

Cell wall biodegradability is strongly influenced by phenolic acids, since certain of the acids are indigestible and even toxic to many soil and ruminal bacteria (Chesson *et al.*, 1982; Borneman *et al.*, 1986; Jung & Sahlu, 1986). However, certain microflora can survive in the presence of phenolic acids and utilise them as a carbon source (McCarthy, 1987). Ferulic acid esterases (FAE) from microbial sources have previously been reported (Johnson *et al.*, 1989), and we have recently purified to homogeneity and partially characterised the enzyme from *Streptomyces olivochromogenes* (Faulds & Williamson, 1991). The pure FAE releases ferulic acid from wheat bran only in the presence of xylanase, showing synergy between enzymes on cell wall material.

The paper examines the activity of FAE against either FAXX (produced from wheat bran) or sugar beet pectin.

EXPERIMENTAL

Enzymes

Streptomyces olivochromogenes (NRCC 2258) was obtained from the National Research Council of Canada Culture Collection, and FAE was purified as previously described (Faulds & Williamson, 1991). The enzyme was assayed using methyl ferulate (Faulds & Williamson, 1991). One unit (U) of activity is defined as the amount of enzyme releasing 1 μmol ferulic acid per minute at pH 6·0 and 50°C.

Pectinase from *Aspergillus niger* was obtained from Sigma Chemical Co. and partially purified using anion-exchange chromatography (Mono Q, Pharmacia) (Fig. 1) using a 0–1 M KCl salt gradient in 20 mM Tris buffer (pH 8·5). Pectinase was assayed as described previously (Williamson *et al.*, 1990). One unit (U) of activity is defined as the amount of enzyme releasing 1 μmol of galacturonic acid per minute at pH 5·0 and 40°C. Protein was estimated using the Coomassie Protein Assay Reagent from Pierce.

Pectin

Sugar beet pectin was extracted from the pulp by heating with acid as previously described (Matthew

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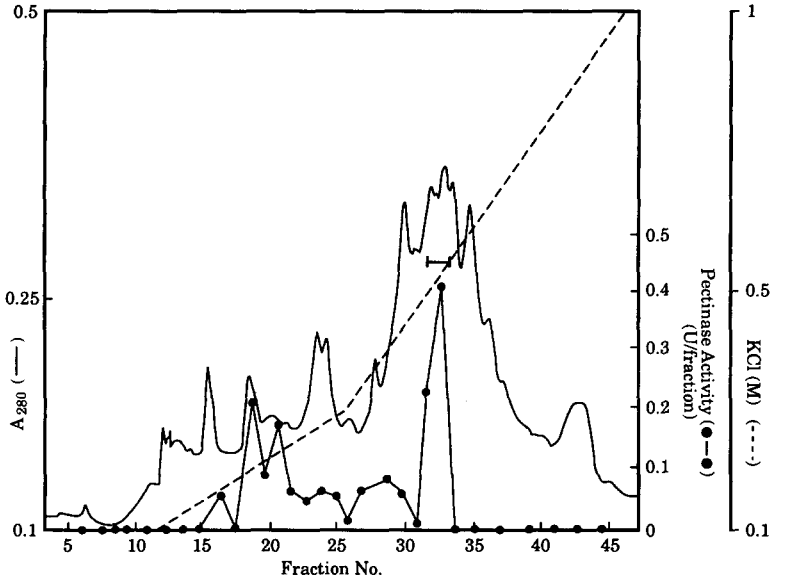

Fig. 1. Elution profile of pectinase on a Mono Q anion-exchange chromatography column. Material was eluted with a 0–1 M KCl gradient in 20 mM Tris (pH 8·5); 1-ml fractions were collected. ----, KCl; ——, A_{280}; ●, pectinase activity; ——, pooled fractions.

et al., 1989). Sugar beet pectin thus extracted has been characterised by a ^{13}C-NMR study (Keenan *et al.*, 1985). Solutions (1% w/v) were prepared by dispersing the polymer in 100 mM 3-[*N*-morpholino]propane-sulphonic acid (MOPS) buffer (pH 6) with gentle heating. Samples were incubated for 21 h at 40°C in the presence of partially purified pectinase (4 U), then incubated for a further 20 h at 50°C in the presence of purified FAE (0·015 mU). Reducing groups were measured by the method of Miller (1959).

De-starched wheat bran

De-starched wheat bran (DSWB) was prepared as previously described (Johnson *et al.*, 1988). It was then milled in a 'shatterbox' (Spex Industries, Inc., Metuchen, NJ, USA) for 180 s generating a fine powder of particle size less than 50 μm. 0-[5-0-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-0-β-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) (Fig. 2) was prepared using the method of Borneman *et al.* (1990); de-starched wheat bran was digested with driselase, a commercial mixture of carbohydrases from Basidiomyces, and the soluble product separated on a Sephadex LH-20 column, in a similar method to the reaction on bamboo. The structure was determined by ^{13}C-NMR and ^{1}H-NMR, and compared to published spectra (Mueller-Harvey *et al.*, 1986). Samples (0·25 μg per ml 100 mM MOPS, pH 6·0) were incubated for 60 min at 50°C in the presence of FAE (0·12 ml). K_m and V_{max} measurements were calculated by the method of Wilkinson (1961) using a 0–2·3 mM FAXX concentration and 0·044 mU FAE (200 μl substrate, 50 μl enzyme).

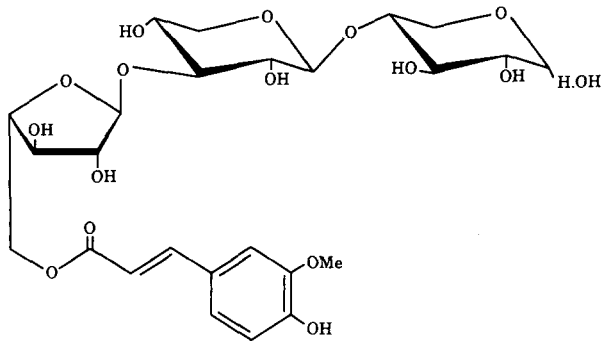

Fig. 2. Structure of FAXX.

RESULTS AND DISCUSSION

When the crude culture supernatant was incubated with de-starched wheat bran, ferulic acid was released at a rate of 0·018 μmol ferulic acid per min per mg protein. However, the purified enzyme failed to release the acid. This was due to the separation of xylanase from FAE during the final step of purification. Adding back the xylanase fraction resulted in the release of ferulic acid in a linear relationship, showing synergy between ferulic acid esterase and xylanase (Faulds & Williamson, 1991).

FAE hydrolysed chemically synthesised methyl ferulate at a rate of 0·19 U per mg protein. This evidence suggests that the enzyme requires low molecular weight substrates for activity, which are produced by the degradation of plant cell walls material by carbohydrases produced by the organism (Faulds & Williamson, 1991).

Hydrolysis of sugar beet pectin

Sugar beet pectin (1% w/v) was incubated with FAE both in the presence and in the absence of an FAE-free pectinase (as measured by methyl ferulate hydrolysis) from *Aspergillus niger*. The presence of pectinase reduces the M_r of the pectin to an average of 4800 showing 4·45% hydrolysis of the pectin polymer. This low M_r material (approximately 25 sugars average in length) is somewhat smaller than the reported size of the 'hairy' region of sugar beet pectin (Rombouts & Thibault, 1986). The hairy region consists of 11 galacturonic acid and 41 neutral sugar residues. Reaction in the presence of both enzymes, over a 40-h period, released 0·25% of the theoretically available ferulic acid (0·155 mg per ml) from the pectin sample. A similar amount was released when FAE was present alone. This suggests that either the hairy region of pectin is too large a complex for FAE to act upon, so that the orientation of ferulic acid amongst the side chains is such that the enzyme cannot attack the majority of the ferulic acid-arabinose ester bond available in the pectin, or that the ferulic acid is linked to a residue for which the enzyme has no affinity.

Hydrolysis of wheat bran FAXX

FAXX as prepared was approximately 66% pure; the contaminant may be another phenolic molecule of an unknown nature. These two components were separated on reverse phase HPLC, and absorbance spectra showed two different compounds, one of which was a feruloyl-containing compound. The K_m and V_{max} of the reaction between FAE and FAXX was calculated to be 0·24 mM FAXX and 0·134 U per mg protein, respectively, at 50°C and pH 6·0. Comparing these values to the K_m of 1·86 mM and V_{max} of 0·3 U per mg protein for the reaction with methyl ferulate, we find that FAE has a higher specificity for FAXX. The purified FAE was also active on the unknown substrate. These results suggest a possible involvement of the sugar moieties in the recognition of the phenolic substance by FAE. The extent of this involvement has yet to be established.

Feruloyl-containing polysaccharides can thus be degraded by a specific esterase acting in conjunction with other carbohydrases to produce low M_r substrates. The action of these enzymes can be used to transform plant polysaccharides into more utilisable forms or to generate economically viable products from waste material.

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